

FUNGAL IMMUNOMODULATORY PROTEIN (FIP) PREPARED BY
MICROORGANISMS AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The invention relates to an improved nucleic acid molecule encoding fungal immunomodulatory protein (FIP) that is better expressed in fungi, to vectors comprising the nucleic acid molecule, to hosts transformed with said vectors, to processes of expressing the protein of the invention in said transformed hosts, to the protein of the invention produced by said processes, to uses of said hosts comprising the protein of the invention and to a process of purifying FIP. The protein of the invention has wide immunomodulatory activity. Thus, the present invention further relates to uses of the protein of the invention in cosmetic or pharmaceutical compositions and to food or feed additives comprising the protein of the invention. Finally, the invention relates to the method of modulating immunological activities by orally administering FIP or proteins fused with FIP to a subject.

Description of the Prior Art

[0002] Lectins from Fungal fruit bodies or mycelia have immunomodulatory and hepatoprotective effects that have been suggested to be able to remove free radicals (Lin J.M. et al., *Am J Chin Med.* 1993;21(1):59-69). (L)-galactoside-specific lectins from mistletoe enhance cytokine production *in vitro* and *in vivo* (Gabijs H.J. et al., *Anticancer Res.* 1992 May-Jun;12(3):669-75). In a test using Balb/C mouse as the disease model, lectins and recombinant lectins can inhibit tumor formation (Couraud P.O. et al., *J. Biol. Chem.* 1989; 264:1310-1316).

[0003] Ganoderma is a rare and valuable herb in Chinese medicine. It has been known in China for over 5,000 years as "Ling Zhi". There are a variety of ganodenmas, including *G. lucidum* (red), *G. applanatum* (brown), *G. tsugae* (red), *G. sinense* (black), and *G. oregonense* (dark brown).

[0004] Several proteins from edible fungi such as *Ganoderma Lucidium* (Ling zhi or Reishi), *Volvariella Volvacea* (Chinese Mushroom), *Flammulina Velutipes* (Golden needle mushroom) share similar amino acid sequences and immunomodulatory functions. These proteins were named fungal immunomodulatory proteins (FIPs) (Ko J.L., *Eur. J. Biochem.* 1995; 228:224-249). Among all health foods in Chinese medicine, Ling Zhi is the best-studied fungus.

[0005] It has been known that Ling Zhi has anti-allergy (Chen H.Y et al., *J. Med. Mycol.* 1992; 33:505-512), hepatoprotective (Lin J.M. et al., *Am J Chin Med.* 1993;21(1):59-69), anti-tumor effects (Wasser SP(1999), *Crit Rev*

Immunol 19:65-96) and immune advantages (Kino (1989), *Journal of Biological Chemistry* 264(1) : 472-8). However, Ling Zhi is used restrictedly in the form of extract of raw material (Horner W.E. et al., *Allergy* 1993; 48:110-116) or small molecules (Kawagishi H., et al., *Phytochemistry* 1993; 32: 239-241).

[0006] One FIP purified from Ling Zhi, LZ-8 (Ling Zhi-8), has positive effects on systemic anaphylaxis (Kino K. et al., *J. Biol Chem.* 1989; Jan 5; 264(1):472-8). LZ-8 has been used for the treatment of liver cancer and to prevent diabetes (Kino K. et al., *J. Biol Chem.* 1989; Jan 5; 264(1):472-8). LZ-8 and another immunomodulatory protein FIP-five obtained from *Flammulina Velutipes* have similar amino acid sequences and folding structures similar to the heavy chain of immunoglobulin. Further, it has been shown that by enhancing the expression of LZ-8, these proteins show immunomodulatory activities and have positive effects on patients with systemic anaphylaxis (Ko J.L., *Eur. J. Biochem.* 1995; 228:224-249).

[0007] Baker's yeast is a safe microorganism widely used in traditional food processing (such as bread, wine). Baker's yeast is the typical model organism to study genetics, physiology and molecular biology. Because of its safety, baker's yeast was also successfully utilized to produce protein drugs such as Hirudin, Hemoglobin, Urokinase, HSA(human serum albumin), IGF-I(insulin-like growth factor 1), GM-CSF(granulocyte macrophage colony stimulating factor), hepatitis B vaccine, etc. (Romanos M.A. et al., *Yeast* 1992; 8: 423-488). The proper host yeast used to produce the designate protein was selected according to the expression profile of the protein expressed. For example, *Saccharomyces cerevisiae* could be used in the production of food and protein drugs.

[0008] At the current stage, LZ-8 can only be obtained from Ling Zhi by a complicated and time-exhausting preparation protocol (such as extraction, US Patent No. 5,334,704). Although there is a great demand of Ling Zhi, natural Ling Zhi is rare and only grows on aged trees in steep mountains. Therefore, a technique to provide constant supply of high quality *Ganoderma* is needed. Currently there is no protocol for massive production of LZ-8 available.

[0009] Using the protocol described in the previous study (US Patent No. 5,334,704), the production of Ling Zhi immunomodulatory protein by extraction and purification is in lower efficiency and requires a higher cost. Other methods using *E. coli* to produce Ling Zhi immunomodulatory protein may have endotoxin contamination and the process of purification still requires a higher cost. Methods using mammalian cells to produce Ling Zhi immunomodulatory proteins require expensive medium and may meet viral or prion contamination.

[0010] US Patent No. 5,334,704 has disclosed that the glycoprotein isolated from ganoderma have immunosuppressive activity. The preparation of the glycoprotein includes steps of culturing ganoderma mycelia, extracting the resultant mycelia with an aqueous solvent and purifying the target protein from the resultant extract. The technique of the patent, which requires growing fungi and purifying the target protein from the fungi, requires a higher cost and is

much more complicated than growing microorganism *in vitro* and is difficult to conduct industrial application.

SUMMARY OF THE INVENTION

[0011] The present invention provides an isolated nucleic acid molecule comprising a nucleic acid sequence as depicted in Figure 1(I).

[0012] The present invention also provides vectors and host cells transformed with vectors.

[0013] The present invention further provides a process for preparing a host cell comprising FIP.

[0014] The present invention further provides the FIP prepared by said processes.

[0015] The present invention further provides uses of said host cells comprising FIP.

[0016] The present invention further provides processes of purifying FIP from said host transformed with said vectors.

[0017] The present invention further provides a composition comprising the FIP of the invention. The composition can be applied to cosmetics, pharmaceuticals, foods and feed additives.

[0018] The present invention further provides a method of orally administering FIP or protein fused with FIP to a subject to modulate its immunological activity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1 shows the nucleotide sequence of the (I) improved FIP codon expressed by *Saccharomyces cerevisiae*, FIP-yeast; (II) the original FIP codon expressed by *Ganoderma lucidum*, FIP-lz.

[0020] Figure 2 shows the nucleotide sequences and restriction sites of (I) the forward and reverse primers of the improved codons of *Saccharomyces cerevisiae*, the FIP-yeast; (II) the forward and reverse primers of the original codons of *Ganoderma lucidum*, the FIP-lz.

[0021] Figure 3 shows the comparison of FIP amino acid sequences expressed in *Ganoderma lucidum*, *Ganoderma tsugae* and *Flammulina velutipes*. Identical amino acids are indicated in bold.

[0022] Figure 4 illustrates the construct map of plasmid (I) pYB101(II) pYB101-FIP-yeast (III) pYB101-FIP-lz.

[0023] Figure 5 shows the quantification of FIP expressed by yeast (DY150 or by4741) transformed with (I) pYB101-FIP-yeast or (II) pYB101-FIP-lz. M: Standard protein showing molecular mass 175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.5 Kd. S: The standard FIP prepared from transformed *E.*

Coli. Lane 1-2: Dy150 yeast No.1-2 transformed with pYB101-FIP-yeast. Lane 3-4: By4741 yeast No.1-2 transformed with pYB101-FIP-yeast. Lane 5-6: By4741 yeast No.1-2 transformed with pYB101-FIP-lz. Lane 7-8: Dy150 yeast No.1-2 transformed with pYB101-FIP-lz.

[0024] Figure 6 shows the nucleotide sequences and restriction sites of (I) the forward and (II) reverse primers of the FIP.

[0025] Figure 7 shows the nucleotide sequence and restriction sites of the -factor leader sequence used to generate pYB101s and pYB101s-FIP. The restriction sites are marked in bold. The beginning of the -factor leader sequence and the FIP gene is marked with an arrow.

[0026] Figure 8 shows the construct map of plasmid (I) pYB101s (II) pYB101s-FIP.

[0027] Figure 9 shows the quantification of FIP expressed by yeast By4741 transformed with pYB101s-FIP. M: Standard protein showing molecular mass 175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.5 Kd. S: The standard FIP prepared from transformed *E. Coli*. Lane 1-3: The first, second and third culture day of By4741 transformed with pYB101s-FIP-yeast.

[0028] Figure 10 shows the western blot of samples of 50L fermentation of yeast comprising FIP. M: Standard proteins. S: Standard FIP generated from *E. Coli*.

[0029] Figure 11 shows the amount of INF- γ generated by peripheral blood cell (PBL) incubated with different concentrations of FIP prepared by different procedures. Blank: PBS control. Batch 1: Supernatant of ruptured yeasts prepared from the first 50L fermentation process. Batch2: Supernatant of ruptured yeasts prepared from the second 50L fermentation process. Yeast: Supernatant of ruptured yeast purchased from the market. PHA: phytohemagglutinin.

[0030] Figure 12 depicts (I) the analysis of protein fractions using ÄKTA™ explorer 10S system. F3, F4 and F5 are the 3rd, 4th, and 5th sample tubes collected. (II) SDS-PAGE of the purified FIP fractions collected by ÄKTA™ explorer 10S system. M: Standard proteins showing molecular mass 175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.5 Kd. STD: The standard FIP prepared from transformed *E. Coli*.

[0031] Figure 13 shows the result of NBT assay on grouper's head-kidney cells under different concentrations of purified FIP. Control: PBS control.

[0032] Figure 14 shows the mortalities of groupers challenged with Iridovirus infection after injecting different concentrations of FIP to their abdomen. PBS: Intraperitoneal injecting 0.1ml PBS before virus challenging.

0.25, 1, 5 ug/0.1ml: Intraperitoneal injecting 0.25, 1, 5 ug/0.1ml purified FIP before virus challenging.

[0033] Figure 15 shows the survival rate of groupers challenged with *Vibrio harveyi* infection after groupers were fed with different amounts of yeast comprising FIP. PBS: Phosphate buffer control. 20mg, 4mg, 0.8mg: Groupers fed with 20mg, 4mg, and 0.8mg yeast comprising FIP. Control: Groupers fed with non-transformed yeast.

[0034] Figure 16 depicts the IFN- γ expression of splenocytes of *Der p* II-sensitized Balb/c mice re-stimulated with FIP or *Der p* II Extract. FIP-gts: FIP. *Der p*: House dust mite Dermatophagoides antigen (Der p2). Control: PBS control.

DETAILED DESCRIPTION OF THE INVENTION

[0035] Since Baker's yeast and Ling Zhi both belong to the fungi kingdom; yeast is expected to perform similar protein modifications (such as glycosylation and disulfide-bond formation) as Ling Zhi when expressing heterologous proteins from Ling Zhi.

[0036] The present invention provides an isolated nucleic acid molecule comprising a nucleic acid sequence as follows:

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ATGTCTGATA CTGCTTTGAT TTTCAGATTG GCTTGGGATG TTAAGAAGTT
GTCTTTTCGAT TACACTCCAA ACTGGGGTAG AGGTAACCCA AACAACTTCA
TTGATACTGT TACTTTCCCA AAGGTTTTGA CTGATAAGGC TTACACTTAC
AGAGTTGCTG TTTCTGGTAG AAAGTTGGGT GTTAAGCCAT CTTACGCTGT
TGAATCTGAT GGTTCCTCAA AGGTAACTT CTTGGAATAC AACTCTGGTT
ACGGTATTGC TGATACTAAC ACTATTCAAG TTTTCGTTGT TGATCCAGAT
ACTAACAACG ATTTTCATTAT TGCTCAATGG AACTGA
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[0037] The present invention further provides the said nuclei acid molecule ligated to an additional signal sequence for secretion. Accordingly, the FIP expressed by host cells, which were transformed with the novel ligation nuclei acid molecule, would be directly secreted into the extracellular fluid and be easily separated from host cells at a lower cost.

[0038] The present invention further provides a ligation gene that can be generated by ligating the said nuclei acid molecule with other genes. Fusion protein composed of different proteins has been generated to provide additive bio-function of both composite proteins (Weisbart RH, *Cancer Lett.* 2003;195(2):211-9.). Since FIP can be absorbed in the digestion system, by fusing FIP with other proteins, FIP can be used as a delivery system to carry other proteins into the target organism. And orally administering the fusion protein should supply benefits of both genes composite of the fusion gene to the target organism.

[0039] The present invention further provides a vector comprising the said nucleic acid molecule. The vector can be transformed into host cells. The vector may be circular or integrated plasmid depending on different uses and conditions.

[0040] The present invention further provides a host cell that is transformed with the vector said. The host cell may be a bacterium, a fungal cell or a yeast cell. The host cell said may be in intact or disrupted form.

[0041] The preferred embodiments of the host cell said are *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Candida utilis*, *Candida boidinii*, *Candida maltosa*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Schwanniomyces occidentalis*, *Schizosaccaromyces pombe*, *Torulopsis*, *Arxula adenivorans*, or *Aspergillus* (*A. nidulans*, *A. niger*, *A. awamori*, *A. oryzae*), *Trichoderma* (*T. reesei*). Cells from plants are also the preferred embodiment of the host cell.

[0042] The present invention also provides a process for preparing a host cell with FIP. The process comprises (a) constructing an expression vector having the improved FIP nucleotide sequence inserted, (b) transforming a host cell with the vector; and (c) culturing the transformed host cell under appropriate conditions. The preferred embodiment of the protein of the invention is depicted in Figure 3. The preferred embodiments of the host cell said are *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Candida utilis*, *Candida boidinii*, *Candida maltosa*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Schwanniomyces occidentalis*, *Schizosaccaromyces pombe*, *Torulopsis*, *Arxula adenivorans*, or *Aspergillus* (*A. nidulans*, *A. niger*, *A. awamori*, *A. oryzae*), *Trichoderma* (*T. reesei*). Cells from plants are also the preferred embodiment of the host cell. The most preferred embodiment of host cell in step (b and c) is *Saccharomyces cerevisiae*. The vectors in step (b) are pYB101-FIP-yeast or pYB101-FIP-lz as depicted in Figure 4(II,III). The FIP codon that was better expressed in fungi (Fig 1(I)) was chosen based on its high tRNA translation efficiency (Sorensen MA, Pedersen S. *J Mol Biol.* 1991; 222(2):265-80).

[0043] The present invention further provides uses of host cells comprising the FIP. For example, intact yeasts comprising the FIP can be orally administered to and absorbed by the target organism without purifying the protein from the host, thereby reducing the cost of utilization.

[0044] The present invention further provides a process of purifying FIP from the said host cells transformed with the said vector, the process comprising (a) dissolving fermented host cells in a solvent; (b) disrupting cells, (c) modify the pH of the solution to pH 4-5; (d) separating debris in the cell extracts; (e) centrifuging the supernatant; (f) adding the supernatant to the column equilibrated to pH 4-5; and (g) eluting the Ling Zhi immunomodulatory protein. In particular, the purification process comprising

steps of (a) dissolving fermented yeasts 1:5-1:20 (v/v) in 5-50 mM PBS (Sigma); (b) disrupting cells in the cell extracts by the disruptor, (c) modify the pH of the solution to pH 4-5; (d) separating debris by centrifuging the solution at 3000g for 15 minutes; (e) centrifuging the supernatant again at 12,000g for 1 minute; (f) adding the supernatant to the CM Sepharose column (Amersham Biosciences) equilibrated to pH 4-5; and (g) eluting the Ling Zhi immunomodulatory protein by adding 20-50mM acetic acid at pH 4-5.

[0045] The present invention also provides the protein of the invention that is prepared by the said process.

[0046] The present invention also relates to the route by which FIP or the host cell comprising FIP is administered to a subject. Administration route may be selected from intravenous, intraperitoneal, oral, mucosa, skin adsorption, or other available routes. Oral administration is the preferred route. For example, host cells comprising FIP can be immersed in solution and FIP will be absorbed to fish through their gills.

[0047] The preferred embodiments of the subject that can be administered with FIP or host cells comprising FIP are mammal, fish, crustacean and poultry. Pig and chicken is the preferred mammal and poultry, respectively. Grouper, salmon and trout are the preferred fish. Shrimp, lobster, *Penaeus monodon*, *Penaeus japonicus* and *Penaeus vannamei* are the preferred crustacean.

[0048] The present invention also relates to a composition for use in modulating immunological activities by oral route comprising fungal immunomodulatory protein. The fungal immunomodulatory protein is prepared from natural Ling Zhi or from the fungal immunomodulatory protein of the process of the invention. According to studies done on the function of the protein of the invention, the said composition could be applied to pharmaceutical use for reducing inflammation and anaphylaxis, modulating immune activity, preventing diabetes, improving asthma, modulating immune responses against bacterial and viral infection, modulating the immune response against organ transplantation; to food or feed additives for prolonging life, modulating immune activity, increasing feed conversion (i.e. the rate of feed weight transferred to body weight) and modulating the negative immune response such as inflammation caused by stress (Black PH, *Brain Behav Immun.* 2003 Oct;17(5):350-64.).

[0049] The present invention further provides a method of modulating immunological disorders by orally administering FIP to a subject. The protein may be prepared from *E. Coli* or fungi such as *Saccharomyces cerevisiae*.

[0050] The invention further provides immunological assays such as western blot or enzyme -linked immunoabsorbant assay (ELISA) to measure the amount of FIP in herbs (such as Ling Zi), foods, beverages, cosmetics, feed additive, or drugs (*Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp: 18.60-18.75.).

[0051] Compositions comprising the protein of the invention may include other agents conventional in the art with regard to the type of formulation. For example, those formulations suitable for oral administration may include other agents such as sweeteners, thickeners and flavoring agents.

[0052] The appropriate composites in the compositions comprising the protein of the invention may depend on the type, severity and stage of disease and vary among different individuals.

[0053] The following examples are intended to illustrate, but not to limit the invention.

Examples

[0054] Example 1: Preparation of Expression Vectors pYB101-FIP-yeast and pYB101-FIP-lz

[0055] Construction of FIP-yeast and FIP-lz

[0056] The DNA sequences of FIP-yeast was depicted in Figure 1(I) and FIP-lz in Figure 1(II). The DNA sequences were prepared according to *Proc. Natl. Acad. Sci. USA*. 1991 May 15;88(10): 4084-4088.

[0057] Preparation of Primers of FIP-yeast and FIP-lz

[0058] The forward and reverse primers of FIP-yeast and FIP-lz were purchased from commercial suppliers (Mission Biotech Co., Ltd. Taiwan) according to the known DNA sequence encoding Ling Zhi immunomodulatory protein (*J. Biol. Chem.* 1991; 266(4), 2486-2493). Restriction sites of *PmlI* and *BamHI* were added to both ends of the primers as depicted in Figures 2(I) and 2(II). PCR reaction was performed to obtain the desired primers. The primers amplified were sequenced and their sequences were confirmed.

[0059] Construction of Expression Vectors pYB101, pYB101-FIP-yeast and pYB101-FIP-lz

[0060] Using techniques currently available, the galactose promoter, gene *Ura 3*, and 2 ori were introduced to pYB (Yeastern Biotech Co., Ltd., Taiwan) to provide a nutrition selection marker and a yeast replication origin, respectively. The new vector, pYB101, as depicted in Figure 4 (I), can be transformed to known Baker's yeast such as BY4741 or DY150. The vector pYB101 and DNA sequences encoding FIP-yeast and FIP-lz were digested by restriction enzymes *PmlI* and *BamHI* (New England Biolabs, Beverly, USA). The digested fragments were purified using PCR Cleaning Up Purification Kit. (Viogene, Taiwan). Three moles of digested fragments of FIP-yeast or FIP-lz were ligated to one mole of digested pYB101 by T4 DNA ligase (New England Biolabs, Beverly, USA) to prepare pYB101-FIP-yeast and pYB101-FIP-lz as depicted in Figure 4 (II) and (III), respectively.

[0061] Transformation of pYB101-FIP-yeast and pYB101-FIP-lz into *E. coli*

[0062] *E. coli* DH5 α were transformed with pYB101-FIP-yeast and pYB101-FIP-lz using standard transformation technique (*Molecular Cloning: A Laboratory Manual*, 2nd Ed. pp: 1.82-1.84). Using commercially available DNA extraction kits (Mini-M Plasmid DNA Extraction Kit, Viogene, Taiwan), large amounts of pYB101-FIP-yeast and pYB101-FIP-lz could be obtained from the transformed *E. coli*.

[0063] Example 2: Preparation of Small Amount of FIP-yeast and FIP-lz protein

[0064] Transformation of pYB101-FIP-yeast and pYB101-FIP-lz into Baker's Yeast

[0065] Baker's yeasts (BY4741 or DY150) were transformed with 1 μ g of of pYB101-FIP-yeast or pYB101-FIP-lz using yeast transformation kit (Yeastern Biotech Co., Ltd., Taiwan).

[0066] Selection of Transformed Yeast By Nutrition Condition

[0067] The transformed yeasts said above were spread and cultivated on YNBD media (0.17% Bacto Yeast Nitrogen Base without Amino Acids (Difco, England), 0.5% ammonia sulfate, 2% glucose, 2% agar, (Sigma, USA)). Using the auxotrophic BY4741 (MAT a his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0) and DY150 (MAT a ura3-52 leu2-3 112 trp1-1 his3-11, 15, ade2-1 can1-100), 0.0024% histidine, 0.0072% leucine, and 0.0012% methionine (BY4741) or 0.0072% leucine, 0.0048% tryptophan, 0.0024% histidine, and 0.0024% adenine (DY150)(Sigma, USA) were added to YNBD media before cultivation. The media was maintained at 30°C for 2-3 days. Uracil was not supplied in the medium to select the transformed yeast. Both vectors pYB101-FIP-yeast and pYB101-FIP-lz possess a gene that encodes uracil. Therefore, transformed baker's yeasts will possess such uracil and are able to survive without providing uracil in the medium, whereas non-transformed yeast cannot survive.

[0068] Testing Transformed Yeasts in Agitator with Small Volume

[0069] The two auxotrophic strains of transformed yeasts said above were inoculated to 50 ml YNBD media added with the required nutrition as described in example 2. The culture was grown at 30°C and 250 rpm for 3 days. The yeast culture was centrifuged and washed 3 times with YNB culture media to remove glucose. The yeast culture was then adjusted to 0.1 OD_{600nm} in 50ml of YNBG culture media (0.17% Bacto Yeast Nitrogen Base without amino acids, 0.5% ammonia sulfate, 2% galactose) with the required nutrition. The yeast culture was grown at 30°C and 250 rpm. The yeast concentration was monitored daily using spectrophotometer Ultrospec 2100 pro (Amersham Pharmacia biotech, USA) to measure the OD_{600nm}, and 1ml of yeast sample was harvested everyday. The sample was centrifuged using Microfuge 18 Centrifuge (Bechman Coulter, USA) at 10000 x g for 5 minutes and was washed 3 times to

obtain the yeast mass. The mass was kept at -20°C.

[0070] Example 3: Quantification of Ling Zhi Immunomodulatory Protein Expressed from Transformed Yeast Using Western Blot Analysis

[0071] The harvested yeast mass were diluted to $OD_{600nm}=0.5$ by PBS (Sigma, USA). The diluted mass was dissolved into 2x SDS sample buffer (Sigma, USA) and heated at 100°C for 3 minutes. After cooling, 10 μ l of the diluted mass were subjected to SDS-PAGE at 100 volts following the protocol described in *Nature* (1970) 227, 680-685.

[0072] Western blot was performed following the standard protocol in *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp: 18.60-18.75. The protein on the gel was transferred to the PVDF membrane (Hybond-P, Amersham Bioscience, USA) by 50mA electrophoresis using Semi-PHOR (Hoefer Scientific Instruments, San Francisco) until all proteins have been transferred. The membrane was then immersed into the blocking buffer at 37°C for one hour and at 4°C overnight. After the removal of blocking buffer, 10⁻³ X rabbit anti-Ling Zhi immunomodulatory protein serum (Taiwan Advance Bio-Pharm Inc., Taiwan) in the blocking buffer were added and incubated with the membrane at 37°C for one hour. After removing the serum solution and washing the membrane with the washing buffer, 10⁻⁴ HRP-labeled mouse anti-rabbit serum (Sigma, USA) was added to and incubated with the membrane at 37°C for one hour. After washing the membrane with washing buffer, color indicator TMB (Sigma, USA) was added to develop the color. To complete the western blot assay, the color reaction was terminated when the color appear by adding water. The final result is shown in Figure 5.

[0073] In Figure 5 (I, II), M and S were standard protein marker and standard FIP purified from *E. coli*. Groups 1-4 were FIP expressed by the improved FIP codon. Groups 2-5 were FIP expressed by the original FIP codon. As shown in Figure 5 (I), groups 1-3 have FIP clearly expressed, whereas no FIP was detected in groups 5-8. This result shows that the improved FIP codon can be highly expressed in yeast.

[0074] Example 4: Preparation of Expression Vectors pYB101s-FIP

[0075] Construction of FIP

[0076] The template sequence of FIP was depicted the sequence of FIP-yeast depicted in Figure 1(I). The DNA sequences were prepared as described in Example 3. Yeast BY4741 was selected as the transform host.

[0077] Preparation of Primers of FIP

[0078] The forward and reverse primers of FIP were purchased from commercial suppliers (Mission Biotech Co., Ltd. Taiwan). PCR reaction was

performed using the pYB101-FIP-yeast (Figure 4) as the template. Restriction sites *PmlI* and *XhoI* were added to the primers as depicted in Figures 6 (I) and (II). PCR reaction was performed to obtain the desired primers. The DNA amplified was sent to sequence (Mission Biotech Co., Ltd. Taiwan) and their sequences were confirmed.

[0079] Construction of Expression Vectors pYB-101s and pYB101s-FIP

[0080] Using techniques currently available, the galactose promoter, gene *Ura 3*, 2 ori, and an α -factor leader sequence (as depicted in Figure 7) were introduced to pYB (Yeastern Biotech Co., Ltd., Taiwan) to provide a nutrition selection marker, a yeast replication origin, and a secretion signal, respectively. The new vector, pYB101s, as depicted in Figure 8 (I), can be transformed to known Baker's yeast such as BY4741 and their sequences were confirmed by sequencing (Mission Biotech Co., Ltd. Taiwan).

[0081] The vector pYB101s and DNA sequences encoding FIP were digested by restriction enzymes *PmlI* and *XhoI* (New England Biolabs, Beverly, USA). The digested fragments were purified using PCR Cleaning Up Purification Kit. (Viogene, Taiwan). Three moles of digested fragments of FIP were ligated to one mole of digested pYB101s by T4 DNA ligase (New England Biolabs, Beverly, USA) to prepare pYB101s-FIP as depicted in Figure 8 (II).

[0082] Transformation of pYB101s-FIP into *E. coli*

[0083] As described in Example 1, *E. coli* DH5 α were transformed with pYB101s-FIP. Large amounts of pYB101s-FIP could be obtained from the transformed *E. coli*.

[0084] **Example 5: Preparation of Small Amount of FIP**

[0085] Transformation of pYB101s-FIP into Baker's Yeast BY4741

[0086] Baker's yeasts BY4741 were transformed with 1 μ g of pYB101s-FIP as described in Example 2.

[0087] Selection of Transformed Yeast By Nutrition Condition

[0088] The transformed yeasts said above were selected from YNBD agar (w/ 0.0024% histidine, 0.0072% leucine, 0.0012% methionine) following the same protocol described in Example 2.

[0089] Testing Transformed Yeasts in Agitator with Small Volume

[0090] The transformed yeasts said above were inoculated to 50 ml YNBD media added with the required nutrition as described in Example 2. The production of FIP protein was induced by adding galactose to the medium, and the secreted FIP was collected everyday.

[0091] **Example 6: Quantification of FIP Expressed from**

Transformed yeast using SDS-PAGE

[0092] The sample FIP protein was analyzed by SDS-PAGE as described in Example 3. The gel was then stained with Coomassie brilliant blue G-250 (Sigma) for 30 minutes, and destained with the destain solution (20% methanol, 10% acetic acid) until the protein band is clear. The staining result is shown in Figure 9.

[0093] Example 7: 50 L Fermentation Process

[0094] Yeast Inoculation in 50 ml Flask.

[0095] Transformed yeasts which produces Ling Zhi immunomodulatory protein were selected by western blot analysis and were inoculated to 50 ml YNBD media together with the required nutrition as in Example 2. The culture was maintained at 30°C and 250 rpm for 24 hours.

[0096] Yeast Inoculation in 2000 ml Flask.

[0097] The 50 ml culture said was inoculated to the same media 450 ml in a 2000 ml flask. The culture was maintained at 30°C and 250 rpm for 24 hours.

[0098] Yeast Fermentation in 50 L Fermentor (Chuan Tai Factory, Taiwan).

[0099] The 500 ml culture said was centrifuged and washed twice with YNB media to remove glucose. The yeast concentration was adjusted to 0.1 OD_{600nm} in 50 L YNBG culture media (0.17% Bacto Yeast Nitrogen Base w/o Amino Acids, 0.5% ammonia sulfate, 2% galactose (Sigma, USA)) together with the required nutrition as in Example 2. The yeast culture was fermented three days at 25-30°C, 400-500 rpm, pH 4.5-5.5. The pH value was adjusted by adding required amounts of 2M NaOH and HCl (Sigma, USA). The pressure in the fermentor was maintained at 0.2-0.4 kg/cm², and 40-50 L/min of ventilation filtrated with 0.2 μm filter.

[0100] Quantification of Ling Zhi Immunomodulatory Protein in 50 L Fermentor

[0101] As in Example 3, some samples were taken from the fermentation culture and the quantity of Ling Zhi immunomodulatory protein in the sample was determined by western blot analysis. As shown in Figure 10, the product from 50 L Fermentor has Ling Zhi immunomodulatory protein.

[0102] Example 8: Activity Assay of Ling Zhi immunomodulatory protein Expressed from Transformed Yeast

[0103] Preparation of Total Cell Extracts

[0104] The harvested yeast mass was washed with PBS and centrifuged 3 times. The yeast pellet was collected and 0.4 mm glass beads of the same

volume of the pellet were added. Mixture was vortexed for 20 sec at 4°C and put on ice for 1 min. This step was repeated five times. After centrifuging at 15,000 rpm for 5 min at 4°C, the supernatant was used as a total cell extract.

[0105] The sample was centrifuged and supernatant was retained. The supernatant was filtrated by 0.2µm membrane (Sartorius) and kept for later use.

[0106] Preparation of Human Peripheral Mononuclear Cells (PMNC)

[0107] Heparinized peripheral bloods were collected from adults and Ficoll-paque culturing media (Amersham Biosciences) was added the blood sample. Following the Amersham protocol, the solution was centrifuged to separate PMNC. Cells were plated at the concentration of 1×10^6 cells/ml in 24-well plate (Nunc, Roskilde, Denmark) for tissue culture. Each 1 ml culture media contains 10 % FBS, 100 µg/ml streptomycin, 100 units/ml penicillin and 200 mM L-glutamate of RPMI1640 medium (GIBCO, Grand Island, NY). Cells were cultivated after growing at 37°C for 0, 24 or 48 hours.

[0108] Cytokine Assay:

[0109] Cells were plated at the concentration of 1×10^6 cells/ml in 24-well plate (Nunc, Roskilde, Denmark) with different concentrations of Ling Zhi immunomodulatory protein added. Cells were cultivated and INF-γ activity was assayed using commercial kit (R&D Systems, Minneapolis, MN). Results are shown in Figure 11. In Figure 11, Batch 1 and Batch 2 were samples added with different concentrations of FIP. PHA (phytohaemagglutinin, Sigma) was used to test INF-γ activity using standard ELISA technique. (*The Journal of Immunology*, 1998, 161: 2114-2119). The blank and yeast groups clearly showed few INF-γ activity, whereas batches 1 and 2, which have purified FIP and PHA added to cells, showed significant INF-γ activity. The results showed that adding 4µg purified FIP to PMNC grown for 48 hours can induce more IFN-γ than adding other proteins. The result also showed samples from 50L fermentation process produce increased amount of IFN-γ, indicating the activity of FIP is not a result of the yeast.

[0110] Example 9: Purification of FIP

[0111] The fermented yeasts were dissolved 1:10 (v/v) in 20 mM PBS (Sigma), pH 6.0. Cells were disrupted by disruptor (Basic Z model (Constant System Ltd. UK)) under 30Kpsi. The pH of the solution was modified to pH 4-5 by adding 1M acetic acid. The solution was centrifuged at 3000g for 15 minutes. The supernatant was separated and centrifuged again at 12,000g for 1 minute. 10mL of the supernatant was added to 30mL of CM Sepharose column (Amersham Biosciences), which was equilibrated with 20-50mM acetic acid to maintain pH 4-5. Adding 20-50mM acetic acid solution, pH 4-5, eluted Ling Zhi immunomodulatory protein.

[0112] From Figure 12 (I), F3, F4 and F5 represents samples from the 3rd, 4th and 5th collection tubes. F3 and F4 clearly had higher mAU value. F3 and

F4 were further analyzed by SDS-PAGE as shown in Figure 12 (II). In Figure 12 (II), F3, F4 and F5 were the 3rd, 4th and 5th collecting tubes. STD represents the standard sample (the purified FIP produced from *E. coli*). M was the standard molecular weight. Proteins in F3 and F4 showed the same molecular weight as the STD. The protein bands in F3 and F4 were further confirmed to be FIP by western blot. This result showed that FIP in F3 and F4 is of very high purity.

[0113] Example 10: The Application of Ling Zhi Protein

[0114] Application of Ling Zhi Protein as Feed Additive.

[0115] Toxicity Test of Ling Zhi Protein

[0116] Groupers with an average weight 5.9g and average length 7.25cm were collected at 25°C and 33 ppt salty degree. FIP purified as in example 9 was intraperitoneally administered to juvenile groupers at the concentrations 5, 1, 0.25µg/fish /0.1ml. PBS solution was administered to the control group. These fish were grown in the original flowing water for 14 days. Fish mortalities were recorded daily. No death was observed in the first two weeks. Therefore, FIP at the above concentrations did not show any toxicity in groupers.

[0117] Enhancement of Nonspecific Immune Response by FIP

[0118] *In Vitro* Enhancement of Nonspecific Immune Response by Treating Grouper's Head-Kidney Cells With FIP

[0119] Six hundred grams groupers were purchased from the market. Head-kidney cells were cultured and phagocytes were separated using standard culture techniques. Head-kidney cells are the main cells affecting nonspecific immune response in groupers (Engelsma MY, *Fish Shellfish Immunol.* 2003 Nov;15(5):397-410). These cells were suspended in AL-10 culturing solution (L15(Gibco) : AIM5(Gibco) = 1:1) and 0.1ml was inoculated at the concentration of 10^7 /ml in 96-well plates (Corning). The plates were maintained at 25 ° C for 2 hours.

[0120] FIP at concentrations 2, 1, 0.5, 0.2 and 0.02 µg/0.1ml were added to the head-kidney culture at room temperature for 2 hours. Nitroblue tetrazolium assay (NBT assay, Sigma) was performed to measure the non-specific immune response. The higher the A_{620nm} value, the higher enhancement of non-specific phagocytosis.

[0121] Adding FIP to the head-kidney cells enhances phagocytosis, which was measured by the NBT assay. As shown in Figure 13, adding 2, 1 and 0.5µg/0.1ml FIP clearly increased the phagocytosis of head-kidney cells, suggesting that FIP can enhance nonspecific immunological response.

[0122] Fish Protection of FIP Against Iridovirus

[0123] Groupers were administered with PBS or FIP intraperitoneally as said in the previous experiment. After growing for three weeks, 0.1 ml of 10,000 x TCID₅₀ viral dosage was administered to the peritoneum of these fish. Fish mortalities were recorded daily. In general, groupers administered intraperitoneally with Iridovirus died in 2 weeks. As shown in Figure 14, fish administered with the highest dosage of FIP, 5µg FIP/fish /0.11ml, showed 70% mortality after two weeks. Therefore, 30% protective rate was achieved in two weeks.

[0124] Fish Protection Against *Vibrio harveyi* Infection.

[0125] One hundred groupers (*Epinephelus malabevicus*, average weight 52.9g) were separated into five groups. Each group included 20 groupers:

1. PBS control;
2. Groupers fed with 20mg original yeast / 60g body weight;
3. Groupers fed with 20 mg FIP-comprising yeast/60g body weight;
4. Groupers fed with 4 mg FIP-comprising yeast/60g body weight;
5. Groupers fed with 0.8 mg of FIP-comprising yeast / 60g body weight;

[0126] Each group was fed once every two days. After 7 days each group were challenged by *Vibrio harvey* infection.

[0127] Groupers were challenged with *Vibrio harvey* at ten times the semi-lethal dosage (LD₅₀) 3X10⁶CFU /ml. Blood samples from 10 fish of each group were collected before the challenge. Nonspecific immunological reactions including lysozyme activity were tested using blood samples said. The other ten fish of each group were challenged with *Vibrio harvey*. The viability, diet and mortality of the fish were recorded daily.

[0128] As shown in Figure 15, after being challenged by *Vibrio Harvey* infection, the 5th-day survival rate of PBS and original yeast groups were significantly reduced to 10-20%; however, the survival rate of three FIP-comprising yeast groups were within 60-80%, suggesting that oral administration of FIP is sufficient to enhance fish immunity and lengthen fish life.

[0129] Example 11 Measured IFN-γ in *Der p*-sensitized Splenocytes from Balb/c Mice Re-stimulated with FIP or *Der p* Extract

[0130] FIP or *Der p* Stimulation

[0131] Spleen cells were collected from two groups of *Der p* (House dust mite Dermatophagoides antigen (Der pII))-sensitized Balb/c mice. Mice of the FIP groups were fed with 2µg of FIP every two days. Mice of the *Der p* groups were administered IP with *Der P* on the first and 7th day and sprayed with *Der p* on the 14th day. Mice were sacrificed on the 15th day and spleen cells were collected.

[0132] Splenocytes isolated from mice were cultured in 5% RPMI-1640 medium at 6×10^6 cells/ml. Splenocytes were then re-stimulated with 10 μ g/ml *Der p* II or 2 μ g/ml FIP. Splenocytes in the control group were added with PBS. Culture supernatants were harvested after 48 hours and the amount of IFN- γ produced was analyzed.

[0133] In Figure 16, *Der p* denoted *Der p* II. FIP-gts denotes FIP. There was no difference of the amount of IFN-r produced between splenocytes from Balb/c mice in the control group and splenocytes from Balb/c mice fed with FIP (81.8 pg/ml). Further, splenocytes from Balb/c mice not fed with FIP or from *Der p* -stimulated mice will produce more IFN-r (463.8 and 1100.7) than the control if re-stimulated with FIP. This experiment demonstrated that FIP could function via oral administration and modulates immunological activity, especially directly inhibiting anaphylactic.

[0134] While the invention has been described and exemplified in sufficient details for those skilled in the art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention.

[0135] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The plasmids, cell lines, uses, processes and methods for producing them are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[0136] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0137] All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[0138] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations, which are not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and

variations are considered to be, within, the scope of this invention as defined by the appended claims.